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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : A61K 35/14, C12P 21/00, 21/02 C12N 15/00	A1	(11) International Publication Number: WO 87/ 07144 (43) International Publication Date: 3 December 1987 (03.12.87)
(21) International Application Number: PCT/US87/01299 (22) International Filing Date: 29 May 1987 (29.05.87) (31) Priority Application Numbers: 868,410 932,767 939,658 (32) Priority Dates: 29 May 1986 (29.05.86) 18 November 1986 (18.11.86) 9 December 1986 (09.12.86) (33) Priority Country: US (71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). (72) Inventors: KAUFMAN, Randal, J. ; 111 Marlborough Street, Apartment #1, Boston, MA 02116 (US). PITTMAN, Debra, D. ; 58 Fairmount Street, Arlington, MA 02174 (US). TOOLE, John, J., Jr. ; 223 High Street, Palo Alto, CA 94301 (US).		(74) Agent: BERSTEIN, David, L.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: NOVEL PROCOAGULANT PROTEINS (57) Abstract Recombinant Factor VIII:c variants, methods to produce the variants and pharmaceutical compositions containing same. The variants of this invention are characterized by modification of one or more specific proteolytic cleavage sites encompassing the arginine residues at positions 220, 226, 250, 279, 282, 336, 359, 562, 740, 776, 1313, 1648, 1719 and 1721; and/or one or both of the lysine residues at positions 325 and 338 and/or serine-741. The variants possess similar or improved procoagulant activity and/or pharmacokinetic profiles compared to that of human Factor VIII:c.		

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NOVEL PROCOAGULANT PROTEINS

Certain aspects of the research resulting in the present invention were funded in part by the U.S. Department of Health and Human Services (DHHS) under a Small Business Innovation Research (SBIR) Grant, DHSS Grant No. 1 R43 HL35946-01. The United States Government has certain rights in this invention.

This invention relates to substances having procoagulant activity. More specifically, this invention relates to "recombinant" procoagulant proteins, a process for obtaining the proteins from genetically engineered cells, and therapeutic compositions containing the proteins for use as procoagulant agents.

The characterization of human factor VIII from plasma indicates that its coagulant activity is associated with a multitude of polypeptide chains having molecular weights ranging from about 50,000 to about 210,000 daltons. Upon addition of thrombin, there is a specified pattern of proteolysis which initially activates and then inactivates the factor VIII procoagulant activity. Definition of the proteolytic cleavages necessary for factor VIII activation and inactivation is required in order to understand the structural requirements for factor VIII activity. One approach has been that of protein sequencing of specific cleavage products before and after digestion with thrombin (Eaton et al., 1986, Biochem. 25:505; Smart et al., 1986, PNAS USA 83:2979-2983). This approach has been somewhat useful in mapping the proteolytic sites for this protease along the factor VIII molecule.

We have now analyzed human recombinant factor VIII derived

from a mammalian host cell system and elucidated the same cleavage sites as determined from plasma derived VIII. Our data suggest that the recombinant protein and the natural protein are folded and processed similarly, a result which could not be predicted with confidence a priori. Our data was obtained using recombinant factor VIII purified from conditioned medium from a mammalian cell line which was engineered to produce factor VIII. The recombinant protein so obtained was characterized as a complex of an approximately 200 kd polypeptide and an approximately 76 kd polypeptide. Upon digestion with thrombin, the 200 kd species yields a 90 kd species with eventual generation of a 50 and a 40 kd species. Upon thrombin digestion the 76 kd species is cleaved to a 69 kd form. The 76 kd and 69 kd species are also referred to elsewhere as the "80 kd" and "73 kd" species. However, the knowledge of the existence of precise cleavage sites has not heretofore definitively established what cleavages are necessary for activation and for subsequent inactivation.

In a further aspect of the research resulting in the present invention, the approach of site-specific mutagenesis coupled with expression of the altered forms of the factor VIII DNA was used to elucidate what sites are necessary and sufficient for the activation, as well as for inactivation of the factor VIII molecule. Specific DNA sequences were changed in order to alter specific amino acids which result in the inactivation of specific cleavage sites. The modified forms of factor VIII were produced using cloned, modified factor VIII-encoding cDNAs in a mammalian host cell system capable of high level expression (Kaufman, PNAS, 1985, 82:689). The modified forms of factor VIII so produced were then analyzed. Our results indicated that a mutation that results in protein that is not cleaved at the 90 kd or 76 kd cleavage site does not

reduce procoagulant activity or thrombin activatability. The predominant species generated in the conditioned medium from the 76 kd cleavage site mutation, at least in the case of deletion variants described hereinafter, is a single chain as monitored by SDS-polyacrylamide gel electrophoresis. Mutation of the thrombin cleavage site generating the 50 and 40 kd species renders factor VIII inactive. Mutation of the proposed activated protein C ("APC") cleavage site at the amino terminus renders factor VIII which has increased specific activity and perhaps decreased susceptibility to proteolytic inactivation. Experimental evidence suggests that APC catalyzes proteolytic cleavage immediately "downstream" of Arg-336 (i.e. between Arg-336 and Met-337) and if cleavage at that site is blocked as described below, perhaps immediately downstream of one or more of Lys-325, Lys-338, and Arg-359.

This invention provides a family of Factor VIII:c-type proteins containing modifications relative to natural human Factor VIII:c which reduce the lability of the molecules for specific protease-catalyzed cleavage at one or more of the cleavage sites of natural human Factor VIII:c but which retain procoagulant activity and thrombin activatability. The sites are referred to hereinafter simply as "cleavage sites" and include the cleavage site between Arg-226 and Ala-227, the "APC" cleavage site including the site between Arg-336 and Met-337 and/or the other proposed "APC" cleavage sites mentioned in the preceding paragraph, the cleavage site between Arg-562 and Gly-563, the "90 kd cleavage site" between Arg-740 and Ser-741, the "95 kd cleavage site" between Arg-776 and Thr-777, the "115 kd cleavage site" between Arg-1313 and Ala-1314, the "76 kd cleavage site" between Arg-1648 and Glu-1649, and the "Factor Xa cleavage site" between Arg-1721 and Ala-1722.

Throughout this disclosure the numbering of amino acids is with reference to the amino acid sequence of Factor VIII:c as depicted in Table 1, wherein the amino terminus of the mature protein is Ala-1.

By "Factor VIII:c-type proteins" (also referred to hereinafter as "variants"), we mean proteins exhibiting factor VIII-type procoagulant activity which are characterized by an amino acid sequence the same or substantially the same, except at one or more cleavage sites, as the amino acid sequence of natural human Factor VIII:c or of analogs thereof (hereinafter, "deletion analogs") containing deletions of 1-1317 amino acids from Ser-373 through Arg-1689, inclusive, which retain procoagulant activity. By an amino acid sequence "substantially the same" as that of natural human factor VIII:c except at one or more cleavage sites we contemplate all factor VIII-type proteins which are characterized by (i) amino acid modification at one or more cleavage sites and either (iia) being encoded by a cDNA capable of hybridizing under stringent conditions to a cDNA which encodes natural human factor VIII:c or (iib) having a mature N-terminal peptide sequence the same or substantially the same as the first 40 amino acids and a C-terminal peptide sequence the same or substantially the same as the last 50 amino acids shown in Table I. Thus, factor VIII-type proteins include full-length and deletion analogs with one or more cleavage site modifications, as described herein, with or without further modification(s), so long as the proteins are active procoagulant or coagulant proteins and either (i) are encoded by a cDNA capable of hybridizing under stringent conditions to a cDNA which encodes a natural human factor VIII:c or (ii) have the same or substantially the same 40 amino acid mature N-terminus and 50 amino acid C-terminus as that shown in Table I. Exemplary further modifications encompassed by

this invention include but are not limited to modifications embodied by "sulfation mutants", i.e. factor VIII:c-type proteins characterized by amino acid substitution for or deletion of tyrosine at one or more potential sulfation sites, e.g. at positions 346, 395, 407, 1664, 1680 and 1709.

The modified forms of factor VIII of this invention may be capable of production in more homogeneous and/or more stable form than serum-derived or recombinant factor VIII and may have beneficial effects upon administration in vivo resulting from increased activity of a single chain molecule, decreased inactivation due to protein C inactivation, increased half-life or specific activity, or improved pharmacokinetic profile. These proteins may thus permit decreased dosages and/or alternative routes of administration relative to unmodified Factor VIII:c.

One aspect of the invention relates to variants wherein one or more of the Factor Xa, APC and thrombin cleavage sites are modified to render such sites less labile to specific proteolysis, for example, wherein one or both of the amino acids defining the cleavage site, preferably at least the arginine residue, is deleted or, as is preferred at present, replaced by a different amino acid. The replacement may be a conservative change, e.g. the replacement of Arg with Lys, to minimize the chance of effecting a change in the secondary structure of the protein. Alternatively the change may be a non-conservative change, e.g. the replacement of Arg with a non-basic amino acid such as Ile, to guarantee resistance to proteolysis. Furthermore, the replacement of a cleavage site amino acid may be with more than one amino acid. For example, an Arg may be replaced with a single amino acid, a dipeptide or a tripeptide such as Ile, Ile-Leu, Leu-Ile,

Table I: Full-length Protein Sequence of Human Factor VIII:c

MQIELSTCFF LCLLRFCFS₁
 A TRRYYLGAVE LSWDYMQSDL GELPVDARFP PRVPKSFPPN₁₀₀
 TSVVYKKTLE VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLKNM ASHPVSLHAV₁₆₀
 GVSYWKASEG AEYDDQTSQR EKEDDKVFPG GSHTYVWQVL KENGPMASDP LCLTYSYLSH₂₂₀
 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD₂₈₀
 AASARAWPKM HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHTFLVRNH₃₄₀
 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME AYVKVDSCPE EPQLRMKNNE₄₀₀
 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPKT WWHYIAAEEE DWDYAPLVLA₄₆₀
 PDDRSYKSQY LNNGPQRIGR KYKKVRFMAY TDETFKTREA IQHESGILGP LLYGEVGD₅₂₀
 LIIFKNQASR PYNIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP₅₈₀
 TKSDPRCLTR YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR NVILFSVFDE₆₄₀
 NRSWYL TENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV FDSLQLSVCL HEVAYWYILS₇₀₀
 IGAQTDFLSV FFSGYTFKHK MVEYEDTLTLF PFSGETVFMS MENPGLWILG CHNSDFRNRG₇₆₀
 MTALLKVSSC DKNTGDYYED SYEDISAYLL SKNNAIEPRS FSQNSRHPST RQKQFNATTI₈₂₀
 PENDIEKTD P WFAHRTMPK IQNVSSSDL MLLRQSPTPH GLSLSDLQEA KYETFSDDPS₈₈₀
 PGAIDSNNSL SEMTHFRPQL HHSGDMVFTP ESGLQLRLNE KLGTTAATEL KKLDKVSST₉₄₀
 SNNLISTIPS DNLAAGTDNT SSLGPPSMPV HYDSQLDTTL FGKKSSPLTE SGGPLSLSEE₁₀₀₀
 NND SKLLESG LMNSQESSWG KNVSSTESGR LFKGKRAHGP ALLTKDNALF KVSISLLKTN₁₀₆₀
 KTSNNSATNR KTHIDGPSLL IENSPSVWQN ILESDETFKK VTPLIHDRML MDKNATALRL₁₁₂₀
 NHMSNKTSS KNMEMVQQKK EGPIPPDAQN PDMSFFKMLF LPESARWIQR THGKNSLNSG₁₁₈₀
 QGPSPKQLVS LGPEKSVEGQ NFLSEKNKVV VGKGFTKDV GLKEMVFPSS RNLFLTNLDN₁₂₄₀
 LHENNTNQE KKIQEEIEKK ETLIQENVVL PQIHTVTGTK NFMKNLFLLS TRQNVESYE₁₃₀₀
 GAYAPVLQDF RSLNDSTNRT KKHTAHFSKK GEEENLEGLG NQTKQIVEKY ACTTRISPNT

(continued---->)

(Table I, continued)

SQQNFVTQRS	KRALKQFRLP	LEETEEKRI	IVDDTSTQWS	KNMKHLTPST	LTQIDYNEKE	1360
KGAITQSPLS	DCLTRSHSIP	QANRSPLPIA	KVSSFPSIRP	IYLTRVLFQD	NSSHLPAASY	1420
RKKDSGVQES	SHFLQGAKKN	NLSLAILTLE	MTGDQREVGS	LGTSATNSVT	YKKVENTVLP	1480
KPDLPKTSBK	VELLPKVHIY	QKDLFPTETS	NGSPGHLDLV	EGSLLQGTEG	AIKWNEANRP	1540
GKVPFLRVAT	ESSAKTPSKL	LDPLAWDNHY	GTQIPKEEWK	SQEKSPKTA	FKKKDTILSL	1600
NACESNHAIA	AINEGQNKPE	IEVTWAKQGR	TERLCSQNPP	VLKRHQREIT	RTTLQSDQEE	1660
IDYDDTISVE	MKKEDFDIYD	EDENQSPRSF	QKKTRHYFIA	AVERLWDYGM	SSSPHVLNR	1720
AQSGSVPQFK	KVVFQEFTDG	SFTQPLYRGE	LNEHLGLLGP	YIRAEVEDNI	MVTFRNQASR	1780
PYSFYSSLIS	YEEDQRQGAE	PRKNFVKPNE	TKTYFWKVQH	HMAPTKDEFD	CKAWAYFSDV	1840
DLEKDVHSGI	IGPLLVCHTN	TLNPAHGRQV	TVQEFALFFT	IFDETKSWYF	TENMERNCR	1900
PCNIQMEDPT	FKENYRFHAI	NGYIMDTLPG	LVMAQDQRIR	WYLLSMGSNE	NIHSIHFSGH	1960
VFTVRKKEEY	KMALYNLYPG	VFETVEMLPS	KAGIWRVECL	IGEHLHAGMS	TLFLVYSNKC	2020
QTPLGMASGH	IRDFQITASG	QYGQWAPKLA	RLHYSGSINA	WSTKEPFSWI	KVDLLAPMII	2080
HGIKTQGARQ	KFSSLYISQF	IIMYSLDGKK	WQTYRGNSTG	TLMVFFGNVD	SSGIKHNIFN	2140
PPIIARYIRL	HPTHYSIRST	LRMELMGCDL	NSCSMPLGME	SKAISDAQIT	ASSYFTNMFA	2200
TWSPSKARLH	LQGRSNAWRP	QVNNPKEWLQ	VDFQKTMKVT	GVTTOGVKSL	LTSMYVKEFL	2260
ISSSQDGHQW	TLFFQNGKVK	VFQGNQDSFT	PVVNSLDPPL	LTRYLRIHPQ	SWVHQIALRM	2320
EVLGCEAQDL	Y					

Ile-Leu-Gly, etc. Compounds of this aspect of the invention thus include variants wherein Arg at one or more of positions 220, 226, 250, 279, 282, 336, 359, 562, 740 (and/or Ser-741), 776, 1313, 1648, 1719 and 1721, is deleted or replaced by one or more amino acids, independently selected from lysine or a non-basic amino acid such as isoleucine, for example. This invention further encompasses Factor VIII:c-type proteins which contain Lys substituted for Arg-1689 at the 69 kd cleavage site, alone or in combination with other modifications described herein. Furthermore, one or both of lys-325 and lys-338 may be deleted or replaced, e.g. with a non-basic amino acid.

Another aspect of the invention relates to variants wherein (a) a tripeptide sequence spanning one or more of the cleavage sites and/or (b) any one or more of the above-mentioned amino acids which may be modified in accordance with this invention is replaced by a consensus asparagine-linked glycosylation site. Consensus N-linked glycosylation sites comprise tripeptide sequences of the formula asparagine-X-threonine or asparagine-X-serine, where X is generally any amino acid except perhaps proline. Exemplary compounds of this aspect of the invention include variants in which the sequence "NRA" spanning the Factor Xa cleavage site is replaced with "NRS" or "NRT". Compounds of this aspect of the invention containing an engineered N-linked glycosylation site at one or more cleavage sites may additionally contain a modification such as arginine and/or lysine deletion or replacement at one or more other cleavage sites, and/or deletion or replacement of tyrosine at one or more sulfation sites, in accordance with the previously-described aspect of the invention. An exemplary compound of this sort contains an Ile substituted for Arg at position 1648 in the 76 kd cleavage site and the

sequence NRS or NRT substituted for NRA at the Factor Xa cleavage site (positions 1720-1722).

One subgenus of variants of particular interest at present includes those containing a modification at the 76 kd cleavage site. These variants thus contain a point deletion or preferably an amino acid substitution at Arg-1648 or a consensus N-linked glycosylation site comprising the sequence -NXT- or -NXS- (wherein X is any amino acid, preferably not proline, however) substituted for Arg-1648, or for QRE, or preferably HQR or REI, the three tripeptide sequences spanning the 76 kd cleavage site. This subgenus includes variants modified only at the 76 kd site, and in addition, e.g. at one, two, three, four, five, six or more other cleavage sites within the purview of this invention and optionally containing Lys instead of Arg at position 1689. For example, this subgenus includes variants in which Arg-1648 is deleted or is replaced with another amino acid or Glu-1649 is deleted or is replaced with Asn, which variants further contain a replacement amino acid for Arg-1313. This subgenus also includes variants modified at one or more of the proposed APC, 90 kd, 95 kd, 115 kd, 76 kd, 69 kd (lysine substitution only) and Factor Xa cleavage sites.

Also, of particular interest at present is the subgenus of variants containing modification at both the proposed APC and Xa cleavage sites. This subgenus also includes variants modified at one or more of the other cleavage sites, including preferably the 76 kd site. Exemplary variants of particular interest at present are depicted in the Table below. Positions marked "X" in the Table indicate the site of deletion of an amino acid or replacement thereof with independently selected replacement amino acids. By "independently selected" we mean that where more than one amino acid position is modified ("X" in

the following Table), replacement amino acids for the respective positions may be the same or different from each other, and one or more of the sites may be modified by deletion while one or more of the other sites may be modified by amino acid substitution. Thus in Compound 19 of the Table below, Arg-740 may be replaced with Ile and Arg-1648 may be replaced with Leu, for example. Alternatively, of course, Arg-740 may be deleted and Arg-1648 replaced with Ile.

Exemplary Variants of this Invention

Factor VIII:C-type proteins* characterized by:

Compound	Deletion or substitution** of:						Xa
	APC				90kd	76kd	
	Arg 336	Lys 325	Lys 338	Arg 359	Arg 740	Arg 1648	R &/or R 1721 1719
1	X						
2	X	X					
3	X		X				
4	X			X			
5	X	X	X				
6	X	X		X			
7	X		X	X			
8	X	X	X	X			
9					X		
10	X				X		
11	X	X			X		
12	X		X		X		
13	X			X	X		
14	X	X	X		X		
15	X	X		X	X		
16	X		X	X	X		
17	X	X	X	X	X		
18						X	
19					X	X	
20	X					X	
21	X	X				X	
22	X		X			X	
23	X			X		X	
24	X	X	X			X	
25	X	X		X		X	
26	X		X	X		X	
27	X	X	X	X		X	
28	X				X	X	
29	X	X			X	X	
30	X		X		X	X	
31	X			X	X	X	
32	X	X	X		X	X	
33	X	X		X	X	X	
34	X		X	X	X	X	
35	X	X	X	X	X	X	
36							X
37	X						X
38	X	X					X
39	X		X				X
40	X			X			X

(continued --->)

(Table, cont'd)

Compound	Deletion or substitution** of:						Xa	
	APC				90kd	76kd		
	Arg 336	Lys 325	Lys 338	Arg 359	Arg 740	Arg 1648	R &/or R 1721	R 1719
41	X	X	X					X
42	X	X		X				X
43	X		X	X				X
44	X	X	X	X				X
45					X			X
46	X				X			X
47	X	X			X			X
48	X		X		X			X
49	X			X	X			X
50	X	X	X		X			X
51	X	X		X	X			X
52	X		X	X	X			X
53	X	X	X	X	X			X
54						X		X
55					X	X		X
56	X					X		X
57	X	X				X		X
58	X		X			X		X
59	X			X		X		X
60	X	X	X			X		X
61	X	X		X		X		X
62	X		X	X		X		X
63	X	X	X	X		X		X
64	X				X	X		X
65	X	X			X	X		X
66	X		X		X	X		X
67	X			X	X	X		X
68	X	X	X		X	X		X
69	X	X		X	X	X		X
70	X		X	X	X	X		X
71	X	X	X	X	X	X		X

*Factor VIII:C-type proteins including full-length FVIII and deletion analogs, including those wherein:

- A-982 through L-1562 is deleted ("DGR")
- S-741 through R-1648 is deleted (90-76kd, "DB")
- S-741 through Q-1647 is deleted (90-R-76kd, "DBR")
- S-373 through R-1648 is deleted (50-76kd)
- T-760 through P-1640 is deleted ("LA")

**The amino acid indicated is either deleted or replaced by a different amino acid, e.g. R -->I or K; K -->I, for example.

Variants in accordance with this invention also include proteins with allelic variations, i.e. variations in sequence due to natural variability from individual to individual, or with other amino acid substitutions or deletions which still retain Factor VIII:c-type procoagulant activity.

All variants of this invention may be prepared by expressing recombinant DNA sequences encoding the desired variant in host cells, preferably mammalian host cells, as is known in the art. DNA sequences encoding the variants may be produced by conventional site-directed mutagenesis of DNA sequences encoding human Factor VIII:c or the deletion analogs thereof.

DNA sequences encoding human Factor VIII:c have been cloned. One sequence encoding the full-length human protein of Table I as well as a sequence encoding the deletion analog pDGR-2 have been deposited under accession number ATCC 53100 with the American Type Culture Collection, (ATCC) in Rockville, MD.

Preparation and nucleotide sequence of the full-length human factor VIII:c cDNA has been set forth in detail in U.S. Patent Applications Serial Nos. 546,650 (filed October 28, 1983) and 644,086 (filed August 24, 1984) and in International Patent Application No. PCT/US84/01641, published May 9, 1985 (Publn. No. WO 85/01961). A pSP64 recombinant clone containing the nucleotide sequence depicted in Table I, designated as pSP64-VIII, is on deposit at the ATCC under Accession Number ATCC 39812.

To prepare cDNA encoding deletion analogs of Factor VIII:c, restriction endonucleases were used to obtain cleavage of the full-length human factor VIII:c cDNA, at appropriate sites in the nucleotide sequence. Restriction

endonucleases are generally utilized under the conditions and in the manner recommended by their commercial suppliers. The restriction endonucleases selected are those which will enable one to excise with substantial specificity sequences that code for the portion of the factor VIII:c molecule desired to be excised. BamHI and SacI are particularly useful endonucleases. However, the skilled artisan will be able to utilize other restriction endonucleases chosen by conventional selection methods. The number of nucleotides deleted may vary but care should be taken to insure that the reading frame of the ultimate cDNA sequence will not be affected.

The DNA sequences encoding the deletion analogs can, in addition to other methods, be derived from the full-length sequence of human factor VIII:c DNA by application of oligonucleotide-mediated deletion mutagenesis, often referred to as "loopout" mutagenesis, as described for example in Morinaga, Y. et al. Biotechnology, 2:636-639 (1984).

Deletion analogs containing a deletion of 1-951 amino acids between the 90kd and 69kd cleavage sites and methods for their preparation are described in detail in co-assigned U.S. Serial No. 725,350 (filed April 12, 1985) and International Application No. PCT/US86/00774 (published 23 October 1986 as WO 86/06101), based thereon. Plasmid pDGR-2 which contains cDNA encoding a deletion analog lacking 581 amino acids has been deposited with the American Type Culture Collection as ATCC 53100. Analogous deletion variants containing a deletion of 1-1317 amino acids between Arg-372 (at the 50/40 cleavage site) and Ser-1690 (at the 69kd cleavage site) can be prepared using the general methods described in PCT/US86/00774, supra. More specifically, a DNA molecule encoding such deletion analogs may be readily prepared from a DNA molecule encoding either

full-length Factor VIII or a previous deletion analog such as pDGR-2, by loop-out mutagenesis using appropriate oligonucleotides or appropriate restriction enzymes, as will be readily understood by those of ordinary skill in this art.

By these means one may readily prepare a cDNA encoding a protein having factor VIII:c type procoagulant activity wherein the protein is characterized by amino acid sequence:

A-X-B

In the formula A-X-B, A represents a protein region comprising the polypeptide sequence Ala-1 through Arg-372 of a full-length sequence of factor VIII:c, e.g. substantially as shown in Table I. B represents a protein region comprising the polypeptide sequence Ser-1690 through Tyr-2332 of a full-length sequence of Factor VIII:c, e.g. substantially as shown in Table I. X represents a protein region comprising 0-1316 amino acids substantially duplicative of sequences of amino acids within the sequence Arg-372 through Ser-1690 of a full-length sequence of Factor VIII:c, e.g. substantially as shown in Table I. It should be understood that the amino terminus of X is covalently bonded through a peptide bond to the carboxy terminus of A, and the carboxyl terminus of X is likewise bonded to the amino terminus of B. It should be further understood, however, that where X represents 0 amino acids, the amino terminus of A is covalently bonded by a peptide bond directly to the carboxyl terminus of B, to form an Arg-372:Ser-1690 fusion. Proteins of this invention may be produced by culturing a host cell containing the appropriate cDNA using conventional expression vectors and techniques. Proteins of this invention include, inter alia, proteins of the formula A-X-B wherein X comprises a peptide sequence of 0-367 amino acids substantially duplicative of sequences of amino acids within the sequence

Arg-372 through Arg-740 of a full-length sequence of factor VIII:c, e.g., substantially as shown in Table I.

As mentioned above, DNA sequences encoding individual variants of this invention may be produced by conventional site-directed mutagenesis of a DNA sequence encoding human Factor VIII:C or deletion analogs thereof. Such methods of mutagenesis include the M13 system of Zoller and Smith, *Nucleic Acids Res.* 10:6487-6500 (1982); *Methods Enzymol.* 100:468-500 (1983); and *DNA* 3:479-488 (1984), using single stranded DNA and the method of Morinaga et al., *Bio/technology*, 636-639 (July 1984), using heteroduplexed DNA. Exemplary oligonucleotides used in accordance with such methods to convert an arginine codon to a codon for isoleucine, for example, are shown in Table II. It should be understood, of course, that DNA encoding each of the proteins of this invention may be analogously produced by one skilled in the art through site-directed mutagenesis using appropriately chosen oligonucleotides.

The new DNA sequences encoding the variants of this invention can be introduced into appropriate vectors for expression in mammalian cells. The procoagulant activity produced by the transiently transfected or stably transformed host cells may be measured by using standard assays for blood plasma samples.

Table II: Exemplary Oligonucleotides

No.	Sequence	Mutation
1.	GTC TTG AAA CGC CAT CAA <u>ATA</u> GAA ATA ACT CGT ACT ACT	R ₁₆₄₈ ----> I
2.	CAT CAA ATA GAA ATA	* (1)
3.	CGC CAT CAA CGG <u>AAC</u> ATA ACT CGT ACT ACT	E ₁₆₄₉ ----> N
4.	CAA CGG AAC ATA AC	* (3)
5.	GCC ATT GAA CCA <u>ATC</u> AGC TTC TCC CAG	R ₇₄₀ ----> I
6.	GAA CCA ATC AGC TTC	* (5)
7.	C TTT ATC CAA ATT <u>ATC</u> TCA GTT GCC AAG	R ₃₇₂ ----> I
8.	CAA ATT ATC TCA GTT	* (7)
9.	GT CCA GAG GAA CCC CAA CTA <u>AAG</u> ATG AAA AAT AAT GAA GCGG	R ₃₃₆ ----> K
10.	CAA CTA AAG ATG AAA	* (9)

(cont'd---->)

Table II (cont'd)

No.	Sequence	Mutation
11.	GAA AAT CAG AGC CCC <u>AAA</u> AGC TTT CAA AAG AAA AC	R ₁₆₈₉ ----> K
12.	AGC CCC AAA AGC TTT	* (11)
13.	CAA CGT AGT AAG <u>ATC</u> GCT TTG AAA CAA TTC	R ₁₃₁₃ ----> I
14.	AGT AAG ATC GCT TTG	* (13)

* Used for screening mutagenesis event effected with the oligonucleotide indicated in parentheses. Codons for replacement amino acids are underlined. As those skilled in this art will appreciate, oligonucleotides can be readily constructed for use in deleting one or more amino acids or for inserting a different (replacement) amino acid at a desired site by deleting one or more codons or substituting the codon for the desired amino acid in the oligonucleotide, respectively. Other mutagenesis oligonucleotides can be designed based on an approximately 20-50 nucleotide sequence spanning the desired site, with replacement or deletion of the original codon(s) one wishes to change.

The eukaryotic cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors such as the bacterial replicons, selection genes, enhancers, promoters, and the like may be obtained from natural sources or synthesized by known procedures. See Kaufman et al., J. Mol. Biol., 159:601-621 (1982); Kaufman, Proc Natl. Acad. Sci. 82:689-693 (1985). Eucaryotic expression vectors useful in producing variants of this invention may also contain inducible promoters or comprise inducible expression systems as are known in the art.

Established cell lines, including transformed cell lines, are suitable as hosts. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants (including relatively undifferentiated cells such as haematopoietic stem cells) are also suitable. Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominantly acting.

The host cells preferably will be established mammalian cell lines. For stable integration of the vector DNA into chromosomal DNA, and for subsequent amplification of the integrated vector DNA, both by conventional methods, CHO (Chinese Hamster Ovary) cells are presently preferred. Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome (Lusky et al., Cell, 36: 391-401 (1984) and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other usable mammalian cell lines include HeLa, COS-1 monkey cells, melanoma cell lines such as Bowes cells, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines and the like.

Stable transformants then are screened for expression of the procoagulant product by standard immunological or activity assays. The presence of the DNA encoding the procoagulant proteins may be detected by standard procedures such as Southern blotting. Transient expression of the procoagulant genes during the several days after introduction of the expression vector DNA into suitable host cells such as COS-1 monkey cells is measured without selection by activity or immunologic assay of the proteins in the culture medium.

Following the expression of the DNA by conventional means, the variants so produced may be recovered, purified, and/or characterized with respect to physiochemical, biochemical and/or clinical parameters, all by known methods.

Wild-type full length factor VIII:c produced in mammalian host cells is characterized by an N-terminal heavy chain of ~200 kD and a C-terminal light chain of ~80 kD together with ~90 kD, ~76 kD and ~69 kD fragments. Upon treatment with thrombin polypeptide fragments including the following are observed by SDS-PAGE: ~180 kD (smear), ~69 kD, ~65 kD (following digestion with factor Xa), ~50 kD, ~45 kD, and ~43 kD. The active species is believed to be a complex of the ~50 kD and ~90 kD polypeptides, perhaps in further association with the ~43 kD polypeptide. In the case of deletion analogs wherein the deletion is between the 90 kD and 76 kD sites, a single chain polypeptide may be observed with a molecular weight of up to ~200 kD, as well as a heavy chain of up to about ~180 kD and a light chain of ~76 kD. After thrombin digestion, a truncated B-domain may be observed of up to ~92 kD, as well as polypeptides of ~69 kD, ~65 kD (after Xa digestion), ~50 kD, ~45 kD and ~43 kD.

By expression in mammalian host cells of factor VIII:c-type-encoding DNA sequences mutagenized to partially or completely abolish specific proteolysis at one or more cleavage sites in the protein in accordance with this disclosure, this invention provides for the first time active factor VIII:c-type procoagulant compositions comprising polypeptides characterized by the following:

(a) the substantial absence of a ~76 kD polypeptide prior and subsequent to thrombin treatment, in embodiments wherein the peptide sequence is modified to prevent cleavage at the 76 kD site;

(b) the substantial absence of a ~65 kD fragment prior and subsequent to factor Xa treatment in embodiments wherein the peptide sequence is modified to prevent cleavage at the Xa site (at arg-1721);

(c) the substantial absence of a ~90 kD fragment prior and subsequent to thrombin treatment in embodiments wherein the peptide sequence is modified to prevent cleavage at the 90 kD site (at arg-740);

(d) the substantial absence of a ~40 kD fragment prior and subsequent to thrombin treatment in embodiments wherein the peptide sequence is modified to prevent cleavage at the APC site (at arg-336); and

(e) combinations of (a) through (d) in embodiments wherein specific proteolysis at more than one of the cleavage sites is prevented.

The proteins of this invention have been found to bind to monoclonal antibodies directed to human Factor VIII:C and

may thus be recovered and/or purified by immunoaffinity chromatography using such antibodies and/or by conventional protein purification methods. Furthermore, these compounds possess Factor VIII:C-like procoagulant activity.

The compounds of this invention can be formulated into pharmaceutically acceptable preparations with a parenterally acceptable vehicle and/or one or more excipients in accordance with procedures known in the art.

The pharmaceutical preparations of this invention, suitable for parenteral administration, may conveniently comprise a sterile lyophilized preparation of the protein which may be reconstituted by addition of sterile solution to produce solutions preferably isotonic with the blood of the recipient. The preparation may be presented in unit or multi-dose containers, e.g. in sealed ampoules or vials. Their use would be analogous to that of human factor VIII, appropriately adjusted for potency.

The invention will be further understood with reference to the following illustrative experimental examples and procedures, which are purely exemplary, and should not be taken as limiting the true scope of the present invention, as described in the claims.

PLASMID DERIVATIONS

The mutagenesis of factor VIII cDNAs was performed directly in the expression plasmid in order to minimize effort in shuffling sequences between different vectors. Generally, the approach taken for mutagenesis was derived from the procedure of Morinaga with modifications. This approach is facilitated by the construction of plasmids which have convenient unique restriction sites in the factor VIII

expression plasmid. The following depicts the construction of a factor VIII expression plasmid which has unique Eco RV, HpaI, Cla I and Xba I restriction sites. Plasmid pMT2 may be obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2VIII was then constructed by digesting pMT2 with Eco RV and XbaI, treating the digested DNA with Klenow fragment of DNA polymerase I, and ligating Cla linkers (NEBiolabs, CATCGATG). This removes bases 2171 to 2421 starting from the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2 (the ClaI derivative of pMT2). The factor VIII cDNA was excised from pSP64 VIII with SalI and blunted with T4 DNA polymerase, and EcoRI adapters were added (AATTCCTCGAGAGCT). The EcoRI-adapted factor VIII cDNA was then ligated into the EcoRI site of the ClaI derivative of pMT2. The resultant plasmid is called pMT2-VIII.

When the full length factor VIII expression plasmid is introduced into COS-1 cells, low levels of factor VIII are obtained. By deletion of a middle region of the factor VIII coding region (See U.S. Serial No. 725,350 and related PCT application, supra) higher levels of Factor VIII were obtained which had characteristics very similar to the native forms of factor VIII including thrombin activability. Thus, the analysis of mutations in factor VIII cleavage sites was facilitated by studying the mutations in these deleted derivatives which are expressed more efficiently. Thus, a deleted form of the factor VIII expression plasmid pMT2VIII was constructed by taking the

KpnI (at 1961 in the factor VIII cDNA) to the XbaI site (in the factor VIII cDNA at 7096 base pairs) from pDGR-2 and ligating it into the KpnI-XbaI fragment of pMT2VIII. The final derivative is pMT2-DGR.

MUTAGENESIS

The mutagenesis of specific sites in the factor VIII expression plasmid involves the following steps:

- 1) The plasmid pMT-DGR was linearized with ClaI, treated with calf intestine phosphatase, and separated on a 0.8% low melting temperature tris-acetate agarose gel. The linearized band was then extracted by adsorption to silica dioxide and eluted in tris-EDTA.
- 2) A second lot of pMT-DGR was digested with KpnI and XhoI or KpnI and XbaI as indicated below, and separated on a 0.8% low melting temperature agarose gel and extracted as above.
- 3) One ug of each of these plasmids were mixed and the volume was adjusted to 18 ul and 2.0 ul of 2 N NaOH was added.
- 4) The mixture was denatured at room temperature for 10 min, then neutralized with 180 ul of a solution which is 0.02 N HCl and 0.1 M Tris-HCl pH 8.0.
- 5) 20 picomoles of phosphorylated mutagenic oligonucleotide was added to 40 ul of the heteroduplex mixture.
- 6) The mixture was placed in a 68°C heat block for 90 min. After the incubation the mixture was allowed to slowly cool at room temperature.
- 7) For each mutagenic reaction, 40 ul of the heteroduplex oligonucleotide mixture was used. The reactions were made 2 mM MgCl₂, 1mM beta-mercaptoethanol, 400 uM ATP, 100 uM deoxynucleotide triphosphate, 3-4 units/ul of Klenow fragment of E. coli DNA polymerase I and 400 units/ul of T4 DNA ligase.

8) The reactions were incubated for 10 minutes at room temperature, transferred to 16°C and incubated overnight.

9) The reaction was terminated by phenol-chloroform extraction and ethanol precipitation, and the resultant pellet was washed with 70% ethanol and resuspended in 10 ul of sterile H₂O.

10) DNA was then used to transform competent HB101 or DH-5 bacteria. The ampicillin resistant colonies were screened with 1x10⁶ cpm/ml of a ³²P-labeled screening oligonucleotide in 5x SSC, 0.1% SDS, 5xdenhardt's reagent, and 100ug/ml denatured salmon sperm DNA.

11) The filters were washed with 5x SSC, 0.1% SDS at a temperature 5 degrees below the calculated melting temperature of the oligonucleotide probe.

12) DNA was prepared from positively hybridizing clones and analyzed initially by digestion with different restriction enzymes and agarose gel electrophoresis. DNA was transferred to nitrocellulose and filters were prepared and hybridized to the screening probes in order to ensure the mutagenic oligonucleotide was introduced into the correct fragment.

13) DNA was then retransformed into E. coli and ampicillin resistant colonies were screened for hybridization to the screening oligonucleotide.

14) Final mutations were confirmed by DNA sequencing (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74:5463-5467).

EXAMPLE 1

Alteration of the 76 kd cleavage site:

The alteration of specific cleavage sites may be accomplished by changing the basic amino acid on the amino terminal side of a potential cleavage site. Since the

choice of amino acid replacement can affect protein folding and/or function the best choices in this regard are conservative alterations. Some proteases, for example thrombin, are very specific for arginine. Thus, alteration of arginine to a lysine may significantly inhibit cleavage. More dramatic modification, for example a change to isoleucine, would guarantee resistance to proteolysis. Since the protease involved in the cleavage of the 76 kd is not known, a change from the arginine at position 1648 to an isoleucine was performed. The mutagenic oligonucleotide was the 39-mer, No. 1 of Table II. The screening nucleotide was the 15-mer, No. 2 of Table II. The mutagenesis was carried out as above with the KpnI-XbaI fragment of pMT2-DGR and the ClaI digested linear form of pMT2-DGR. The resultant mutant was demonstrated to be correct by DNA sequencing (Sanger et al., supra). DNA (pCSM 1648) was prepared by banding in CsCl and used to transfect COS-1 monkey cells as described (Kaufman, PNAS, 1985, 82:689). 60 hr. post transfection, samples of the conditioned media were taken for factor VIII activity assay by the Kabi Coatest chromagenic assay method (Kabi) or the ability to clot factor VIII deficient plasma (Activated Partial Thromboplastin Time, APTT) before and after thrombin activation. Results from the activity assays are shown in Table III. The mutation of the 76 cleavage site did not decrease the activity of factor VIII generated in the conditioned media. In addition, there was no change in the thrombin activation coefficient. In order to demonstrate that the mutation did actually destroy the cleavage site, the transfected cells were labeled with ³⁵S-methionine for 6 hrs and conditioned media and cell extracts prepared for analysis by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The results demonstrated that the alteration of Arg to Ile did not affect the synthesis or secretion of the factor VIII

variant from the cell. Analysis of the radiolabeled protein after thrombin digestion indicated a normal appearance of the 69 kd, and 50 and 40 kd fragments. However, the predominant factor VIII species produced was a single chain molecule as a result of resistance to cleavage at the 76 site. This result demonstrated that single chain factor VIII is as active as the native molecule. The single-chain Factor VIII:c variants may be advantageous in that they may be produced in more homogeneous form and may have an improved pharmacokinetic profile relative to natural human or other recombinant Factor VIII:c proteins.

EXAMPLE 2

An alternative to the arg-->ile change at the 76 kd cleavage site was to introduce an N-linked glycosylation site at asparagine adjacent to the arg in order to attempt to block cleavage. The potential advantage of this alteration is that the resultant protein would have a carbohydrate moiety to potentially block the modified amino acid from provoking an immunologic response. Thus mutagenic oligonucleotide No. 3 of Table II was synthesized. This mutagenesis event converted a Gln-Arg-Glu-Ile-Thr sequence to Gln-Arg-Asn-Ile-Thr. The oligonucleotide used for screening for the mutation was the 14-mer. No. 4 of Table II. For this mutation, the mutagenesis was done in the native, not deleted, factor VIII cDNA which was cloned into a single stranded phage M13 vector. The Sall fragment containing the entire factor VIII cDNA was inserted into the Xho I site of the M13 origin vector, pGC2. pGC2 is a plasmid containing ampicillin resistance, an E. coli origin of replication, an M13 origin of replication and a polylinker containing a XhoI site. Other similar, commercially available plasmids may also be used, of course. The phosphorylated (20

pMoles) mutagenic oligonucleotide was annealed in 10 ul with 1 ug of template containing 20mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, at 65°C for 10 min. The reaction was slowly cooled and 10 ul of solution B [20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM of each nucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 10 mM ATP, 400 units/ml ligase and 3-4 units/ul of Klenow fragment of DNA polymerase I], incubated 5 min at 23°C and then incubated overnight at 16°C. The reaction was terminated by phenol-chloroform extraction and ethanol precipitation. The DNA was resuspended in 10 ul of 10mM Tris-HCl pH 7.5 and 1mM EDTA, and 1 ul taken to transform E. coli. HB101.

DNA (CSM-1649) was prepared and transfected into COS-1 cells as above. After transfection of COS-1 cells as before the conditioned media was assayed and found to contain a relatively low level of activity similar to that produced by the wild-type Factor VIII:c cDNA in pMT2. Analysis of ³⁵S-methionine labeled protein as above indicated that the addition of the N-linked sugar partially blocked cleavage. The ability of this particular type of mutation to block cleavage and allow secretion will probably vary from one protein sequence to another depending on the structure of the protein.

EXAMPLE 3

Mutation of the 90 kd cleavage site:

The mutation of the arginine to an isoleucine at position 740 was performed with oligonucleotide No. 5 of Table II. The correct mutations were screened with 15-mer No. 6 of Table II. The mutagenesis was performed with the KpnI-XbaI

fragment of pMT2-DGR and the ClaI-digested linear form of pMT2-DGR. The resulting DNA (CSM-740) was prepared and transfected as described above. Samples were assayed as described above and CSM-740 was found to generate less activity than pMT2-DGR. Analysis of ³⁵S-methionine labeled cell extracts and conditioned media by immunoprecipitation and gel electrophoresis indicated that Factor VIII synthesis, secretion, activity, and thrombin activation were not dramatically modified by alteration of this cleavage site. Close inspection indicated a less efficient level of secretion for CSM-740. Thus, cleavage of the 90 kd cleavage site is not essential for factor VIII activity.

EXAMPLE 4

Mutation of the thrombin cleavage site at 372:

A. The mutagenic oligonucleotide to convert an arginine to an isoleucine at position 372 was oligonucleotide No. 7 of Table II. The oligonucleotide used to identify correct mutations was No. 8 of Table II. The mutagenesis was carried out with the KpnI-XhoI fragment of pMT2-DGR and the ClaI digested linear form of pMT2-DGR. The resultant plasmid DNA (CSM-372) was prepared and transfected into COS-1 cells as described above. Samples were assayed as above. The results demonstrated that destruction of the 372 cleavage site results in a loss of more than 90% of factor VIII activity. In addition, thrombin treatment does not restore activity. Further analysis indicated that the modified form of factor VIII was properly synthesized and secreted.

B. To produce the K-372 variant, Example 4A may be repeated using analogs of oligonucleotides 7 and 8 of Table II which contain a Lys codon, e.g. AAA, instead of the Ile codon ATC.

EXAMPLE 5

Mutation of the thrombin cleavage site at R-336 (the proposed activated protein C cleavage site):

A. The mutagenic oligonucleotide to convert an arginine to a lysine at position 336 was oligonucleotide No. 9 of Table II. The oligonucleotide used to screen the mutations was No. 10. The mutagenesis was carried out with the KpnI-XhoI fragment of pMT2-DGR and the ClaI digested linear form of pMT2-DGR. The resultant DNA (CSM-336) was prepared, transfected, and resultant samples assayed as above. The results indicate increased activity and a normal thrombin activatibility. The modified factor VIII was not affected in its synthesis or secretion. The increased activity may be attributable to loss of inactivation as a result of proposed Xa cleavage in the cobas assay. Thus, this alteration appears to generate a more stable form of factor VIII.

B. To produce the I-336 variant Example 5A was repeated using analogs of oligonucleotides 9 and 10 of Table II which contain an Ile codon, e.g. ATC, instead of the Lys codon AAG. The I-336 variant so produced had similar biological properties to those of the K-336 variant. Additionally, full-length I-336 and K-336 variants were produced and found to possess similar biological properties to those of the corresponding mutant deletion variants.

EXAMPLE 6

Mutation of the 69 kd cleavage site:

A. The oligonucleotide for mutagenesis of the arginine to a lysine was No. 11 of Table II. The screening oligonucleotide was the 15-mer No. 12 of Table II. Mutagenesis

was performed with the KpnI-XbaI fragment of pMT2-DGR and the ClaI digested linear form of pMT2-DGR. DNA harboring the correct mutation (CSM- 1689) was prepared and transfected into COS cells. Cells were analyzed as above. Results indicate that mutation of the 69 kd cleavage site results in similar activity to that generated by pMT2-DGR. Thus, our lysine-for-arginine mutation at the 69 kd cleavage site does not destroy Factor VIII:c activity.

B. To produce the I-1689 variant Example 6A was repeated using analogs of oligonucleotides 11 and 12 of Table II which contain an Ile codon, e.g. ATC, instead of the Lys codon AAA. Surprisingly, the I-1689 variant so produced was found to possess less than 90% of the Factor VIII:c activity obtained with pMT2-DGR. Our results suggest that cleavage at the 69 kd site is important in activating the molecule and that substitution of Lys for Arg-1689 does not abolish such cleavage. Furthermore, K-1689 variants may be useful therapeutically, perhaps with delayed onset of Factor VIII:c activity.

Although the majority of these mutations were constructed and analyzed in the deleted form of factor VIII (DGR), the alterations can be made directly with DNA encoding full-length factor VIII or can be reintroduced from mutagenized deletion variant DNA into the full length factor VIII cDNA by digestion of mutagenized deletion variant DNA and DNA encoding w.t. Factor VIII:c with the appropriate enzymes and ligation of the appropriate fragments to generate the desired plasmids. In addition, a similar approach can be used to introduce multiple mutations into the factor VIII cDNA. In every case tested we have found that results obtained with mutagenized deletion variants were also obtained with the corresponding full-length variants and that the effect of making multiple amino acid substitutions

may be additive with respect to the separately observed results for particular amino acid modifications. Variants containing amino acid modification at both the proposed APC cleavage site, e.g. at R-336, and the Xa cleavage site at R-1721 should be particularly stable variants that are resistant to inactivation.

TABLE III

Activity of modified forms of factor VIII expressed in COS-1 cells:

<u>Mutation</u>	<u>Activity mU/ml</u>	<u>Thrombin Activation</u>
Experiment 1		
CSM-336 R->K	431	10-fold
CSM-372 R->I	10	-----
CSM-740 R->I	114	10-fold
CSM-1648 R->I	246	10-fold
pMT2-DGR	288	10-fold
Experiment 2		
CSM-1649 E->N	196	10-fold
pMT2VIII	185	10-fold
Experiment 3		
CSM-1689 R->K	88	N.T.
pMT2-DGR	103	N.T.

N.T. = Not Tested

What is claimed is:

1. A protein having human Factor VIII:c-type procoagulant activity and an amino acid sequence substantially that of human factor VIII:c or a deletion analog thereof containing a deletion of 1-1317 amino acids between Arg-372 and Ser-1690, characterized in that (i) arginine-1689 is replaced with lysine and/or (ii) one or more amino acids selected from the group consisting of

- (a) the arginine at one or more of positions 220, 226, 250, 279, 282, 336, 359, 562, 740, 776, 1313, 1648, 1719 and 1721;
- (b) one or both lysines at positions 325 and 338;
- (c) one or more tyrosines at positions 346, 395, 407, 1364 and 1380; and,
- (d) serine at position 741

is deleted or replaced with an independently selected replacement amino acid.

2. A protein of claim 1, characterized in that a tripeptide sequence encompassing one or more of positions 220, 226, 250, 279, 282, 325, 336, 338, 359, 562, 740, 741, 776, 1313, 1648, 1719 or 1721 is replaced with a tripeptide sequence comprising Asn-X-Thr or Asn-X-Ser, wherein X is any amino acid.

3. A protein of claim 2, wherein X is not Arg.

4. A cDNA encoding a protein of claims 1-3.

5. A host cell containing a cDNA of claim 4 operatively linked to an expression control sequence and capable of expressing the protein encoded by the cDNA.

6. A method for producing a protein having human Factor VIII:c-type procoagulant activity and an amino acid sequence substantially that of human factor VIII:c or a deletion analog thereof containing a deletion of 1-1317 amino acids between Arg-372 and Ser-1690, characterized in that arginine-1689 is replaced with lysine and/or one or more amino acids selected from the group consisting of

- (a) the arginine at one or more of positions 220, 226, 250, 279, 282, 336, 359, 562, 740, 776, 1313, 1648, 1719 and 1721;
- (b) one or both lysines at positions 325 and 338;
- (c) one or more tyrosines at positions 346, 395, 407, 1364 and 1380; and,
- (d) serine at position 741

is deleted or replaced with an independently selected replacement amino acid, said method comprising culturing a host cell of claim 5 under conditions permitting production of the protein.

7. A protein produced by the method of claim 6.

8. A pharmaceutical composition for treating or preventing Hemophilia A which comprises an effective amount of a protein of claim 1 in admixture with a parenterally acceptable vehicle or excipient.

9. A pharmaceutical composition for treating or preventing Hemophilia A which comprises an effective amount of a protein of claim 7 in admixture with a parenterally acceptable vehicle or excipient.

10. A protein having factor VIII:c type procoagulant activity characterized by an amino acid sequence:

A-X-B

wherein region A represents the polypeptide sequence Ala-1 through Arg-372 substantially as shown in Table I; region B represents the polypeptide sequence Ser-1690 through Tyr-2332 substantially as shown in Table I; and region X represents a peptide sequence comprising 0-367 amino acids substantially duplicative of sequences of amino acids within the sequence Arg-372 through Arg-740 of Table I, wherein the amino terminus of X is covalently bonded through a peptide bond to the carboxy terminus of A, and the carboxy terminus of X is likewise bonded to the amino terminus of B.

11. A cDNA encoding a protein of claim 10.

12. A host cell containing a cDNA of claim 11 operatively linked to an expression control sequence and capable of expressing the protein encoded by the cDNA.

13. A method for producing a protein having factor VIII:c type procoagulant activity characterized by an amino acid sequence:

A-X-B

wherein region A represents the polypeptide sequence Ala-1 through Arg-372 substantially as shown in Table I; region B represents the polypeptide sequence Ser-1690 through Tyr-2332 substantially as shown in Table I; and region X represents a peptide sequence comprising 0-367 amino acids substantially duplicative of sequences of amino acids within the sequence Arg-372 through Arg-740 of Table I, wherein the amino terminus of X is covalently bonded through a peptide bond to the carboxy terminus of A, and

the carboxy terminus of X is likewise bonded to the amino terminus of B, said method comprising culturing a host cell of claim 12 under conditions permitting production of the protein.

14. A pharmaceutical composition for treating or preventing Hemophilia A which comprises an effective amount of a protein of claim 10 in admixture with a parenterally acceptable vehicle or excipient.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01299

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(4) : A61K 35/14, C12P 21/00, C12P 21/02, C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S.	435/68, 70, 172.3, 253, 317; 424/101; 530/383

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

Computer Search CAS, BIOSIS, APS under: Factor VIII, clone, gene, sequence, DNA, RNA, plasmid, deletion.

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A	M.A. TRUETT ET AL., "Characterization of the polypeptide composition of human factor VIII:C and the nucleotide sequence and expression of the human kidney cDNA", <u>DNA</u> , Volume 4, Number 5, pages 333-349, published 1985 by Mary Ann Liebert Inc. (New York, New York), See the entire document.	1-14
A	R.M. LAWN; The molecular genetics of hemophilia: Blood Clotting Factors VIII and IX", <u>Cell</u> , Volume 42, pages 405-406 published September 1985 by MIT Press (Cambridge, Mass., USA), see the entire document.	1-14
A	D.C. LYNCH ET AL., "Molecular cloning of cDNA for human von Willebrand Factor: Authentication by a new method", <u>Cell</u> , Volume 41, pages 49-56, published May 1985, by MIT Press, (Cambridge, Mass., USA), see the entire document.	1-14

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²

14 AUGUST 1987

Date of Mailing of this International Search Report ³

24 AUG 1987

International Searching Authority ¹

ISA/US

Signature of Authorized Officer ²⁰

Robin Lyn Teskin
Robin Lyn Teskin

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
A	D.N. FASS ET AL., "Internal duplication and sequence homology in factors V and VIII", <u>Proc. Natl. Acad. Sci. Vol. 82, pages 1688-1691</u> , published March, 1985, by the National Academy of Science (Washington, D.C., USA), See entire document.	1-14
Y,P	R.L. BURKE ET AL., "The functional domains of coagulation factor VIII:C", <u>J. Biol. Chem., Volume 261, Number 27, pages 12574-12578</u> , published 25 September 1986, by the American Society of Biological Chemists, Inc. (Washington, D.C., USA) See pages 12575-12578 in particular.	1-14
A	K. TITANI ET AL., "Amino acid sequence of human von Willebrand Factor", <u>Biochemistry Volume 25, pages 3171-3184</u> , published 1986, by American Chemical Society, (Washington, D.C., USA) See the entire document.	1-14
A	D. EATON ET AL., "Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, Factor XA, and activation of factor VIII coagulant activity", <u>Biochemistry Volume 25, pages 505-512</u> , published 1986, by American Chemical Society, (Washington, D.C., U.S.A.), See the entire document.	1-14
A	J.E. SADLER ET AL., "Cloning and characterization of two cDNA's coding for human von Willebrand factor" <u>Proc. Natl. Acad. Sci. Volume 82, pages 6394-6398</u> , published October 1985, by National Academy of Science (Washington, D.C., U.S.A.), See the entire document.	1-14
Y,P	J.J. TOOLE ET AL. "A large region (~ 95 KDa) of factor VIII is dispensable for <u>in vitro</u> procoagulant activity" <u>Proc. Natl. Acad. Sci. Volume 83, pages 5739-5942</u> , published August 1986, by National Academy of Science, (Washington, D.C., U.S.A.) See the entire document.	1-14

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

- | | | |
|---|---|------|
| A | D. BONTHRON ET AL., "Nucleotide sequence of pre-pro-von Willebrand factor cDNA", <u>Nucleic Acids Research</u> , Volume 14 Number 17 pages 7125-7127, published 1986, by IRL Press (Oxford, England), See the entire document. | 1-14 |
| A | C.L. VERWIEIJ ET AL., "Construction of cDNA coding for human von Willebrand factor using antibody probes for colony-screening and mapping of the chromosomal gene", <u>Nucleic Acids Research</u> , Volume 13, Number 13, pages 4699-4717, published 1985, by IRL Press (Oxford, England), see the entire document. | 1-14 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

MICROORGANISMS

Optional Sheet in connection with the following microorganisms referred to in the description:

<u>plasmid</u>	<u>accession number***</u>	<u>1st referred to on page/line</u>	<u>date of deposit</u>
pDGR-2*	53100	13/19-20	12 Apr 1985
pSP64-VIII	39812	13/28-31	23 Aug 1984
pMT2-VWF**	67122	23/3-5	29 May 1986

* in E. coli HB101

**in E. coli DH-5

***deposits made in accordance with Budapest Treaty

Name of depository institution

AMERICAN TYPE CULTURE COLLECTION

Address of depository institution

12301 PARKLAWN DRIVE, ROCKVILLE, MD 20852 USA
telephone: (301) 881-2600

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☒ The date of receipt (from the applicant) by the International Bureau is

17 AUGUST 1987

(17. 08. 87)

Ch. GRASSIOULET

(Authorized Officer)